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Award Number:

W81XWH-11-1-0280

TITLE:

Laser raman tweezer spectroscopy (RTS) for the molecular and functional characterization of single live mouse mammary tumor initiating cells (TIC).

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CONTRACTING ORGANIZATION:

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REPORT DATE:

April 2013

TYPE OF REPORT:

Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                        Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-42013	Final	15 Aug 2011 – 14 Aug 2013

4. TITLE AND SUBTITLE
Laser Raman Tweezer Spectroscopy (RTS) for the Molecular and

Cellular Characterization of Mouse Mammary Tumor Initiating Cells (TIC)

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0613

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Kansas Medical Center
Kansas City, Ks 66160

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The **main goal** of this proposal is to assess whether Raman Spectroscopy (RS) combined with optical tweezer technology (RTS) can be utilized in order to identify distinct subpopulations of highly tumorigenic cells among an otherwise homogeneous population of tumor initiating cells derived from a p53 null mouse mammary tumor model. Laser activated Raman backscattered light from cells generates spectral profiles (wavelengths). The spectral profiles represent molecular and biochemical signatures or intracellular molecular profiles (IMPs) of living cells. Our **working hypothesis is** that tumor-initiating cells (lineage^{-} CD24^{hi}/CD29^{hi}) derived from p53null mouse mammary tumors contain distinct subpopulation of cells with various tumorigenic potential. RTS, by intracellular molecular profiling, would allow one to distinguish heterogeneity among an otherwise homogeneous population of tumor initiating cells (TICs). **Methods:** Individual TICs were sorted by flow cytometry, placed on a quartz slide (high thermal conductivity) and radiated for RS measurements. Laser beam power was optimized to maximize Raman signature and minimize cell damage. **Significant Findings:** RS identified distinct subpopulations within the TICs derived from individual p53 null mouse mammary tumors. We have demonstrated that Raman Spectroscopy (which identifies cells with unique intracellular molecular profiles) combined with flow cytometry (which identifies cells with unique extracellular surface molecules) may be utilized in order to distinguish heterogeneity among an otherwise homogeneous population of TICs. RS induced minimal damage to the cells and there was minimal interference on RS measurement by the quartz slide. Single live tumor initiating cells generated profiles in the wavelength of 600-1800 watts. Future aims are to isolate cells derived from the distinct subpopulations within the TICs and to assess their in vitro and in vivo tumorigenic potential.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			
U	U	U	UU	14	19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION:

The purpose of this research is to utilize Raman Spectroscopy (RS) combined with optical tweezer technology (RTS) to isolate and characterize the intracellular molecular profiles of live tumor initiating cells (TICs) derived from a p53 null mouse mammary tumor model. The rationale is that previous technology has used flow cytometry and antibodies to specific extracellular markers for the purpose of isolation and characterization of tumor initiating cells (TICs). RTS would allow characterization of cells based on their intracellular molecular profiles. Combining the methods of flow cytometry with RTS would allow one to distinguish cells with distinct intracellular molecular profiles among an otherwise homogeneous population of TICs and to translate the distinct intracellular molecular profiles to in vitro and in vivo biological functions such as tumorigenicity. The long-term goal of this project is the isolation and characterization of distinct tumor initiating cells; which would enable future development of anti-cancer stem cell therapies for eradicating resistant and or quiescent TICs.

Task 1. To determine the tissue culture condition, microscope lens setting, and laser intensity for obtaining Raman spectra of live single cells derived from p53 null mouse mammary tumors with minimal damage to the cells.

We initially used a breast cancer cell line, SUM225. The rationale was that SUM225 cells were large (15-30 micron) enough to be visualized by an upright microscope at various, 4 to 100 X, magnifications. Visualizing the cells at various magnifications enabled the optimization of laser intensity with minimal damage to the cells. We were able to obtain Raman spectra using a 100X lens of a confocal microscope submerged into the tissue culture medium and by the use of a HeNe laser (632.8 nm) excitation. The scattered Raman signal was detected on a Peltier-cooled, back-illuminated, deep depletion CCD chip (Andor, Northern Ireland) after passing a holographic transmissive grating. Integration time was 20 seconds repeated three times and the mapping interval was 2 microns. We established that tissue culture medium that contained phenol red and culture plates made of plastic interfered with Raman spectra. By switching to Quartz slides and using phosphate buffer saline (PBS), background Raman signal was minimized. We have also been successful in obtaining Raman signals from single live TICs sorted from p53null mouse mammary tumors (**Figure 1**). **Figure 2** shows that, similar to SUM225, there is no visible cell damage following two 2.5 minutes (total=5 minutes) Raman acquisition period using the HeNe laser illumination to the TICs derived from p53null mouse mammary tumors.

Task 2. To perform RS profiling of single live cells within the tumor initiating cells (TICs) derived from p53 null mouse mammary tumors.

2a.   Isolation of TICs from p53 mouse mammary tumors:
A highly tumorigenic p53null mouse mammary tumor model was obtained from the laboratory of Dr. Jeffrey M. Rosen at Baylor College of Medicine and was used in these studies. BALB/c female mice (3- to 5-weeks old) were injected with pieces of p53 null mouse mammary tumors (1-3mm in diameter) into cleared fat pads. All mice developed mammary tumors at the injection site. The mice were humanely sacrificed 17-21 days following injection on days of Raman analysis. Each p53 null mammary tumor was excised and underwent digestion for 2.5 hours per established protocols in the laboratory. After the initial excision, the tumors remained separate throughout the digestion protocol. Single cells were obtained and prepared for flow cytometry by staining with particular antibodies in order to discern the specific cell population in question. In order to sort 500,000 cells, at least 20 million cells underwent staining. Primary antibodies, including APC/Cy7 anti-mouse/rat CD29 and PE Rat anti-mouse CD24, as well as antibodies for lineage (Lin): Biotin anti-mouse CD31, Biotin anti-mouse CD140a, Biotin Rat anti-mouse CD45 BD, and Biotin Rat anti-mouse Ter-119 were used in conjunction with a secondary antibody of APC Streptavidin in accordance with the accepted staining protocol within the laboratory. After sorting for the TICs identified by $Lin^{-}CD24^{hi}CD29^{hi}$ population (**Figure 3**), the TICs were then pipetted onto a quartz microscope slide, allowed to adhere (10-15 minutes), then bounded by a pap-pen in circular form. We then covered the cells with 15-20µl of PBS to be analyzed by a 100x immersion lens on the Raman microscope. Prior to usage with the immersion objective, a 10x graphic depicting the positioning of the adherent cells was illustrated in order to locate specific cells undergoing Raman scanning.

2b.   Raman experimental setup and measurements
We recorded spectra by scanning individual cells under an integrated fully automated confocal Raman microscope (Jobin Yvon LabRam ARAMIS Micro-Raman Microscope). The imaging system was equipped with two sources of internal laser excitation (HeNe: 632.8nm, Diode: 785nm), a polarization analyzer, and a computer-controlled heating/cooling stage (set at room temperature). A 100x water immersion microscope objective was used in the experiments. Spectra were recorded at an average laser power of 3 mW measured at the microscope objective. The nominal spectral resolution was about ~8 cm-1. The quartz object slide was clamped to the microscope stage and PBS was continually added to the pap-pen circle every 15 minutes to combat evaporation. In this way, the cells could be kept in good condition for longer than the acquisition time of an image scan.

When charting the composition of adherent cells, one must ensure that the contribution of the substrate to the signal is minimal but the Raman scattering from the cells is maximal. For this reason, we initially acquired x,z map (depth scans) to determine the correct distance above the substrate for x,y imaging (typically at z ≈ 2µm). In addition, Raman spectral profiles can be corrupted by the activity of cosmic rays which decrease image quality. To combat the presence of cosmic rays within an acquisition, we used a 100x immersion lens instead of 60x in the 600-1800 cm-1 spectral range. Furthermore, we acquired two 2.5-minute scans to provide a five minute measurement in total for each cell. As a result, if one scan displayed cosmic rays and the subsequent scan did not, the averaged scan excluded the abnormality altogether.

2c.   Data analysis

The data analysis aims to identify intra-tumoral heterogeneity based on the Raman scans. VisuMap software allowed us to interpret the data, helping to explore and understand patterns, relationships, and correlations present within the Raman profiles. Quantitative data was first imported into VisuMap. An initial RPM (Relational Perspective Mapping) Map was retrieved with an accompanying Correlation algorithm. VisuMap's multi-dimensional scaling allowed a specific coordinate to "float" and find another of similar character. In other words, the three-dimensional coordinate system coupled to each Raman scan is not fixed or predetermined, permitting truer association among data points (TICs represented by their Raman scan). From RPM we had the ability to toggle between principal component (PC) analysis to the representative multi-dimensional scale. The ability to switch between analysis features helped us to normalize the data in any number of ways, including similarities and differences involving the intensity and wavelength of profile features. We also supplemented RPM and Correlation algorithm with a clustering strategy called Greedy Agglomerative Clustering (GAC). The GAC algorithm starts with a set of clusters that contains each data point and uses an exhaustive searching algorithm to progressively merge similar smaller clusters into larger clusters according to the original Correlation algorithm in use.

As shown in **Figure 4**, distinct subpopulations were identified within the TICs derived from an individual tumor by Relational Perspective Mapping (RPM) algorithm followed by three dimentional mapping. Each tumor is color coded to demonstrate the intra-tumoral heterogeneity. RPM is based on the correlation algorithm, helping to group TICs according to their quantitative similarities and differences in Raman Spectra.

Figure 5, illustrates the heterogeneity in Raman Profiles of the distinct populations within the TICs in an each tumor. Based on these data, we conclude that there is not only intra-tumoral heterogeneity but also inter-tumoral heterogeneity with respect to the distinct sub-populations of tumor initiating cells and RS may be used to isolate and characterize the tumorigenic subpopulation of TICs within an individual tumor.

Task 3. To utilize RTS for isolating p53 null derived tumor initiating cells (TICs) with distinct intracellular molecular profiles. The TICs with distinct intracellular molecular profiles will be evaluated for their in vitro and in vivo tumorigenicity.

Figure 6 is a diagram describing our future plans for the isolation and assessment of tumorigenicity of the distinct populations identified within the TICs that possess distinct RS (identified as the various gated regions within **Figures 4 & 5**). Please note that a Raman Tweezer technology is currently not available at our institution. As demonstrated in **Figure 6**, TICs are sorted by flow cytometry. The cells are then placed on quartz slides and RS obtained as described previously. Individual cells with a distinct RPM will be taken off the slide by a micropippetor. The individual groups of cells that exhibit distinct intracellular molecular profiles by RS will be assessed for their in vitro and in vivo tumorigenicity. In vitro tumorigenic potential will be assessed by cell motility and invasion assessed by the modified boyden chamber assay, proliferation index by Edu incorporation and mammosphere assays. These assays are established methods used routinely in the laboratory. In vivo tumorigenicity will be also performed by cleared fat pad transplantation. It has been shown that cells in Lin$^-$CD24^{hi}CD29^{hi} subpopulation generate tumors with a frequency of 1 in 27 cells (95% CI: 1/42-1/18) (Zhang, 2008). Therefore, various cell concentrations (50, 25, 10, 5, and 1) from 5-7 identified groups (that display distinct profiles) and a control group (randomly isolated) will be injected into cleared fat pads of 6 mice or 12 fat pads per cell dilution (225 mice total including 25% loss factor). Poisson statistics will be performed to calculate statistical differences in frequency of TIC. It is expected that at least one group will display higher TIC frequency compared to the total population of TIC (Lin$^-$CD24^{hi}CD29^{hi}).

**KEY RESEARCH ACCOMPLISHMENTS:**

- Oral Presentation at The University of Kansas Lawrence, Bioengineering Research Center, Lawrence, Kansas, April 12, 2012.

- An abstract was submitted to the 2013 Mammary Gland Biology Gordon Conference and was selected by the meeting organizers for oral presentation. Daniel Neuman who is a pre-med student working on this project will give the presentation at the Mammary Gland Gordon Biology Conference to be held from June 9-14, 2013 at Stowe flake Resort and Conference Center in Stowe, VT.

- Upon the completion of future plans (depicted in **Figure 6**), we will submit a manuscript reporting our results by the end of this year in 2013.

REPORTABLE OUTCOMES:

We have demonstrated that Raman Spectroscopy (which identifies cells with unique intracellular molecular profiles) combined with flow cytometry (which identifies cells with unique extracellular surface molecules) may be utilized in order to distinguish heterogeneity among an otherwise homogeneous population of TICs.

CONCLUSION:

We have shown that it is feasible to obtain Raman signals from individual live cells derived from the TICs of p53null mouse mammary tumors. We have established that Raman signals may be obtained from cells that have adhered to a quartz slide and are excited by a HeNe laser. We have also demonstrated that the TICs display distinct intracellular molecular profiles as shown by their unique RS. Based on the current data, we conclude that there is not only intra-tumoral heterogeneity but also inter-tumoral heterogeneity with respect to the distinct sub-populations of tumor initiating cells and RS may be used to isolate and characterize the tumorigenic subpopulation of TICs within an individual tumor. Future goals are to obtain data from large quantities of sorted p53 null mouse mammary TICs (ie 30-50 cells per TIC population per tumor). The cells that map to the individual regions with unique spectra will be assessed for in vitro and in vivo tumorigenicity as depicted in **Figure 6**. The future directions will also be to discern, 1) do the TICs isolated from each region (**Figure 7; a-d**) define a distinct subpopulation of cells with certain level of tumorigenic potential regardless of which tumor the cells were derived from, or 2) do the TICs from each region define a distinct tumorigenic subpopulation of cells and the level of tumorigenic potential is specific to each individual tumor.
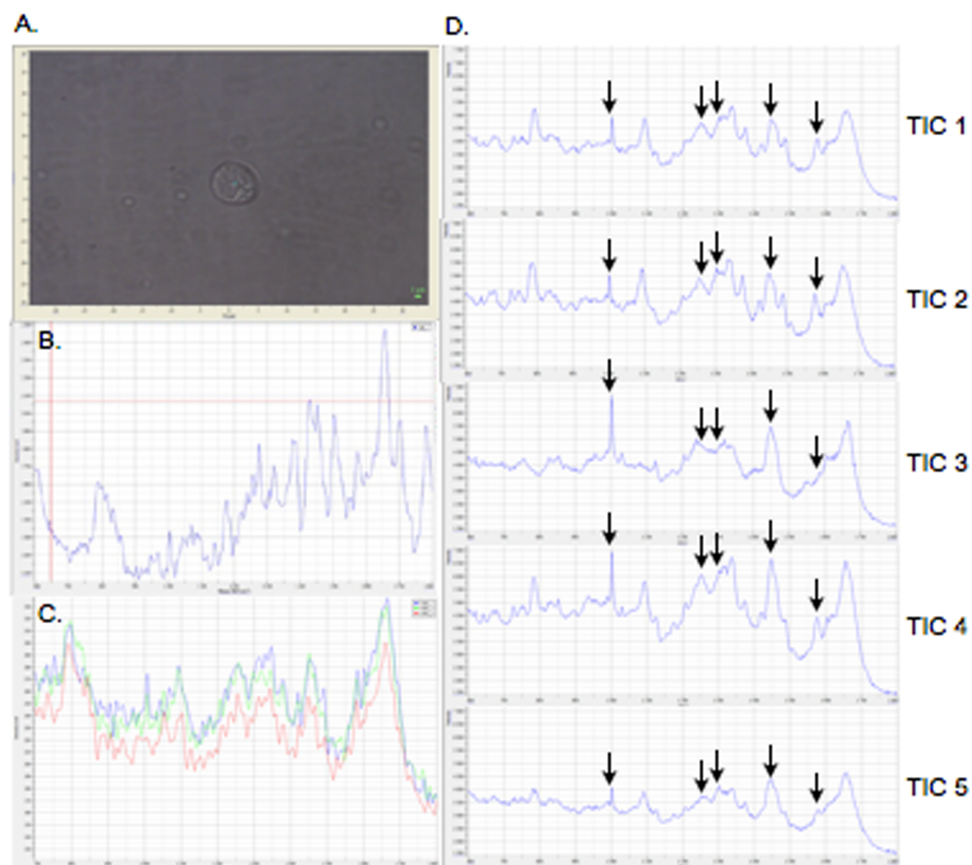
Figure 1. Raman spectra of single live tumor initiating cells (TIC) from a p53 null mouse mammary tumor model. A. Brightfield image of one single live TIC used for acquiring Raman fingerprints. B. Raman shifts observed in the 600-1800cm^{-1} regions. C. Raman shifts following scanning of three TICs. D. Raman shifts observed in five TICs derived from p53 null mouse mammary tumors. Arrows indicate regions of heterogeneity among the TIC at 1000, 1250, 1300, 1450, and 1575 cm^{-1}.
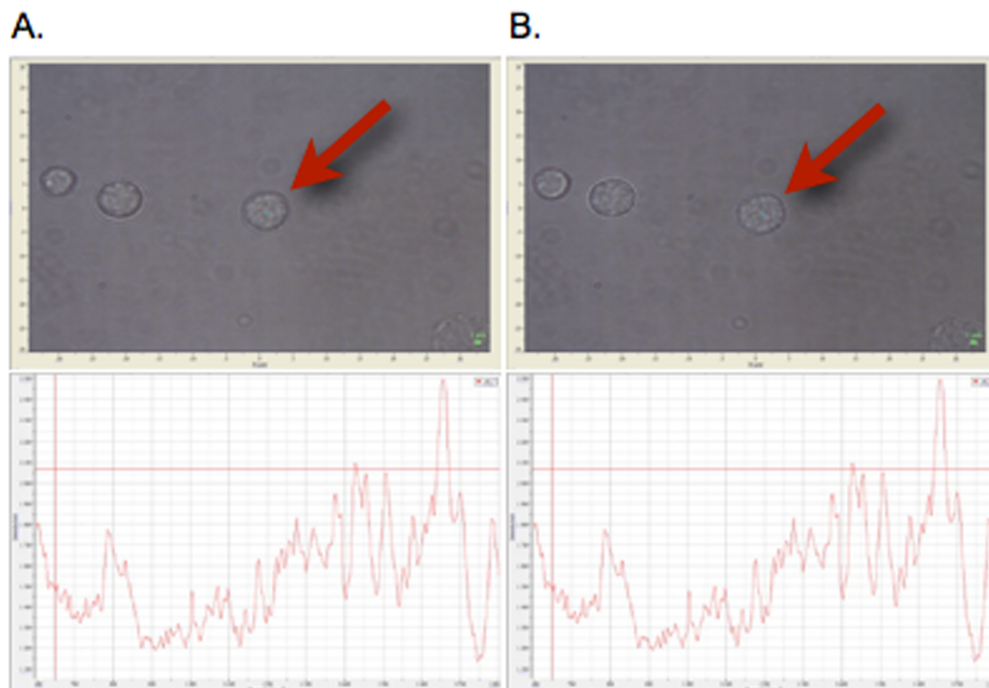
Figure 2. A representative averaged Raman spectrum of a single live TIC of a p53 null mouse mammary tumor. A HeNe laser (632.8nm) is used for excitation at a 1μm resolution over 2.5-minute acquisition periods. The arrows point to a brightfield image of a single live TIC before (A) and after (B) obtaining Raman spectra. As seen, there is no visible sign of cell damage or change in Raman spectrum following a 5-minute acquisition period.
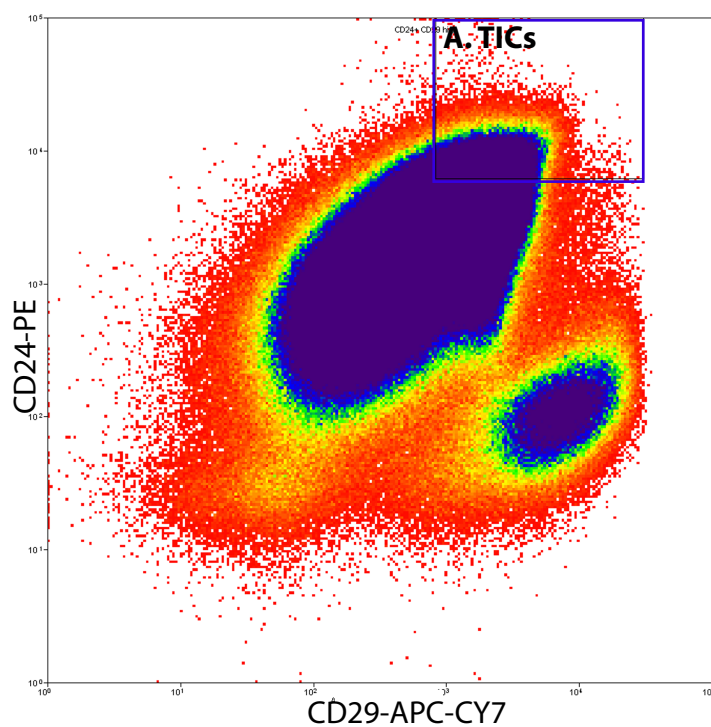

Figure 3. Flow cytometry analysis followed by sorting of TIC subpopulation that were subsequently scanned via Raman microscopy. (A) The target region gated for the TICs are Lineage-CD24hiCD29hi.
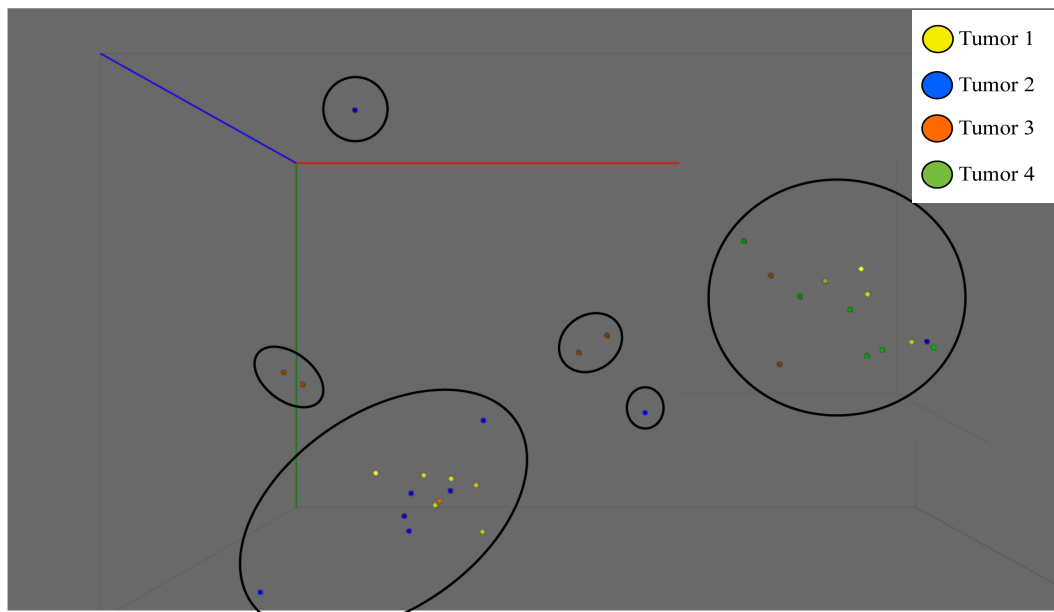
Figure 4. Three dimentional mapping after performing the Relational Perspective Mapping (RPM) algorithm to form distinct groups of cells within an individual tumor. Each tumor is color coded to demonstrate that TICs show distinct popualtions within an individual tumor as measured by RPM. RPM is based on the correlation algorithm, helping to group TICs according to their quantitative similarities and differences. The circles within each view represent the main groups seen after the running of RPM and Correlation algorithm.
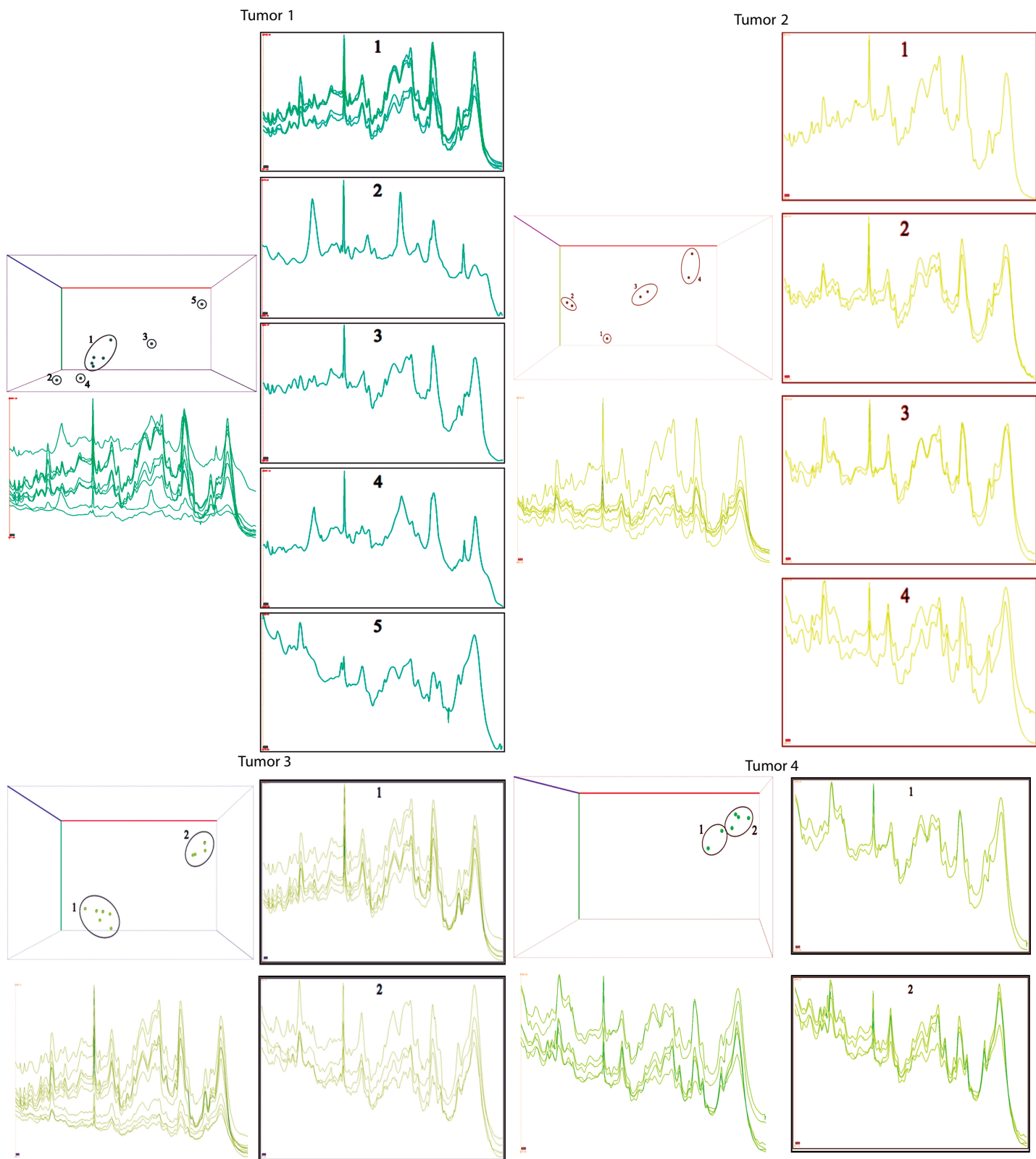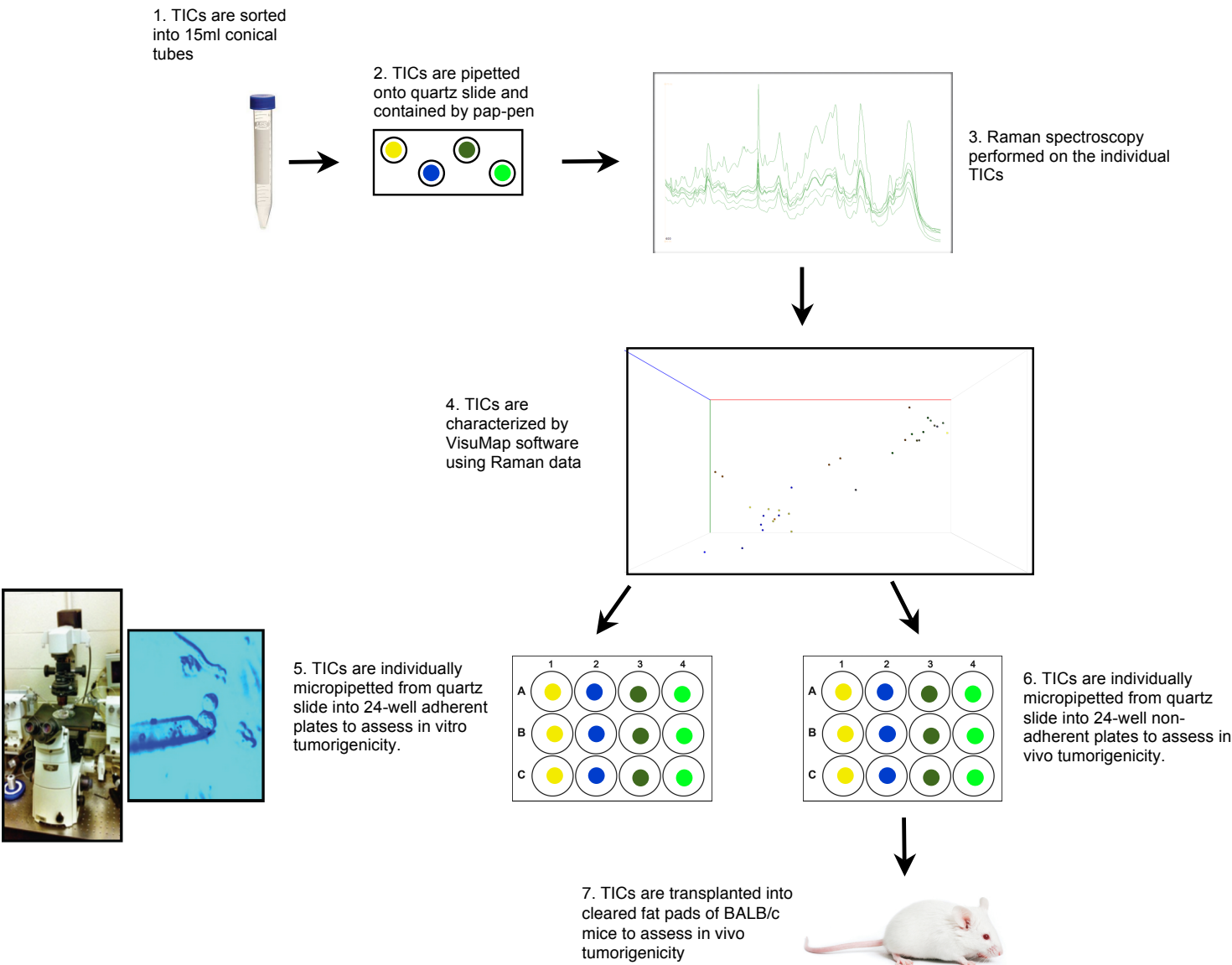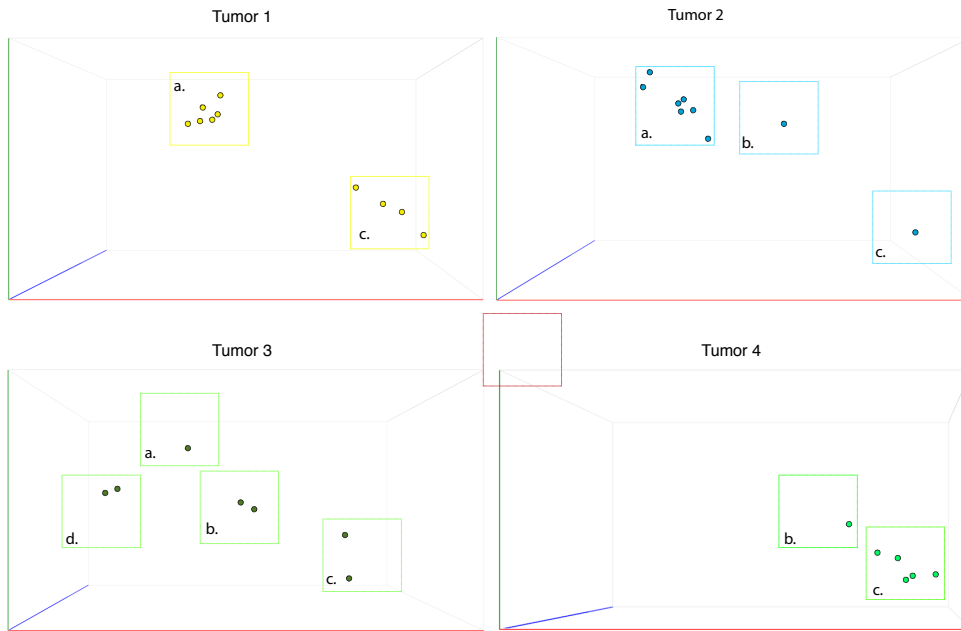
Figure 5. Raman spectra of distinct groups of TICs within each individual tumor (Tumors 1-4). The spectrum number on the right column corresponds to its respective group seen in the 3D map. Below the 3D map is a Raman spectrum containing all groups (all TICs from that single tumor) together. In addition to forming groups of TICs from multiple tumors (Figure 4), Relational Perspective Mapping (RPM) also forms distinct TIC groups within each tumor. The varying patterns and intensities of Raman spectra between groups further highlights the presence of inter-tumoral and intra-tumoral heterogeneity.

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Figure 6. Future Plans

1. TICs are sorted into 15ml conical tubes

2. TICs are pipetted onto quartz slide and contained by pap-pen

3. Raman spectroscopy performed on the individual TICs

4. TICs are characterized by VisuMap software using Raman data

5. TICs are individually micropipetted from quartz slide into 24-well adherent plates to assess in vitro tumorigenicity.

6. TICs are individually micropipetted from quartz slide into 24-well non-adherent plates to assess in vivo tumorigenicity.

7. TICs are transplanted into cleared fat pads of BALB/c mice to assess in vivo tumorigenicity

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A.

Tumor 1   Tumor 2
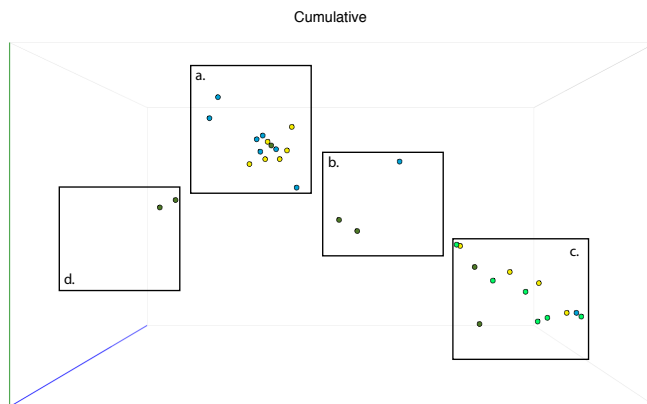
Tumor 3   Tumor 4

B.

Cumulative

Figure 7. A Hypothetical Model.   A. As illustrated each individual tumor's TICs contain subpopulations with distinct Raman Spectra. When the tumors are grouped together as seen in B., distinct subpopulations are similarly observed. The future directions will be to discern, 1) Do the TICs isolated from each region (a-d) define a distinct subpopulation of cells with certain level of tumorigenic potential regardless of which tumor the cells were derived from, 2) Do the TICs from each region define a distinct tumorigenic subpopulation of cells and the level of tumorigenic potential is specific to each individual tumor.

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Zhang, M., Behbod, F., Atkinson, R. L., Landis, M. D., Kittrell, F., Edwards, D., Medina, D., Tsimelzon, A., Hilsenbeck, S., Green, J. E., et al. (2008). Identification of tumor-initiating cells in a p53-null mouse model of breast cancer. Cancer Res 68, 4674-4682.

APPENDICES:
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